Original Article
Salvianolic acid A exhibits anti-tumor activity in nasopharyngeal carcinoma cells through regulation of Pin1

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Abstract: Background: Nasopharyngeal carcinoma (NPC) remains as a devastating malignancy due to a lack of effective therapy for recurrence and metastasis. Salvianolic acid A (SAA), a compound extracted from *Salvia miltiorrhiza*, possesses a variety of biological properties such as antioxidant, antithrombotic, myocardial ischemia protection and anti-hepatic-fibrosis. However, the effect of SAA on NPC cells has not been clarified yet. Methods: NPC cell lines and immortalized nasopharyngeal epithelial cells NP69 were treated with SAA at different concentrations (0-40 μM). Series of relevant experiments were carried out to test the effect of SAA on the proliferation and migration ability of NPC and NP69 cells Pull-down assay and Western blot were used to find out the possible molecular target of SAA. Results: Our results indicated that SAA could inhibit the proliferation of NPC cells in a dose and time dependent manner. And SAA was able to weaken the clone-forming ability as well. Meanwhile, the migratory and invasive capabilities were also attenuated. Moreover, SAA didn't exhibit any cytotoxicity on immortalized nasopharyngeal epithelial NP69 at the same treatment condition. Furthermore, high concentration of SAA could promote the apoptotic of CNE2 and HNE1 cells dramatically. The results of *in vitro* pull down assay showed that SAA might bind with Pin1 directly. And the results of western blot indicated that SAA could down-regulate the expression level of cyclin D1, p53, p21 and Bcl-2, which are downstream substrates of Pin1. Conclusions: SAA exhibits anti-tumor activity in NPC through down-regulating Pin1 and it may be a potential drug for NPC.

Keywords: Salvianolic acid A (SAA), nasopharyngeal carcinoma (NPC), Pin1

Introduction

Nasopharyngeal carcinoma (NPC) is a type of malignant tumor commonly occurred in South Eastern Asia, especially in South-Eastern China such as Guangdong and Hong Kong [1]. According to a report, there were estimated 86,700 new cases of NPC and 50,800 deaths in 2012 worldwide [1]. The etiology of NPC is broadly speculated to be associated with Epstein-Barr virus infection, environmental and genetic factors [2-4]. Radiotherapy was the primary treatment for this cancer. However, more than 30% of patients would experience relapse either with loco-regional recurrence or distant metastases after radiotherapy or chemo-irradiation [5]. What's worse, these treatments were inclined to cause serious adverse reactions and induce multidrug resistance (MDR). Hence, it is of great urgency to identify highly effective anti-NPC drugs with less serious adverse effects.

Salvia miltiorrhiza (SM, also called Danshen) is a well-known traditional Chinese medicine, which has been widely used in Asian and Western countries for the treatment of various diseases, especially for heart and brain diseases [6, 7]. Salvianolic acid A is a water-soluble compound which is extracted from SM. It is one

of the most effective components of SM and its' structure is very simple. It has been reported that SAA owns a number of pharmacological functions such as antioxidant [8], antfibrotic [9], antiplatelet and antithrombotic activities [10]. In a word, SAA is regarded as a potential drug with high efficacy, low toxicity, and low cost. Recent studies have found that SAA might be applied to oncotherapy. Researchers discovered that SAA could inhibit proliferation, cause cell cycle arrest at the S phase, and induce apoptosis in breast cancer cells and lung cancer cells [11, 12]. At the same time, SAA also acted as a supporter to conventional anti-cancer drugs in oncotherapy [13-15]. In this study, our datas indicate that SAA exhibits proliferation-inhibiting, migration-suppressing and apoptosis-promoting effects on NPC cells through suppressing the activity of peptidyl-prolyl isomerase Pin1.

Methods and materials

Reagents and materials

Salvianolic acid A (SAA, ≥98% purity) powder was purchased from Sigma-Aldrich (Shanghai). A stock solution of SAA was made at 10 mM in ddH$_2$O and stored at -20°C. CNE2, HNE1, and NP69 cells were obtained from our research center. Fetal bovine serum (FBS), RPMI-1640 medium, Keratinocyte-SFM and 0.25% trypsin were purchased from life technologies (Grand Island, NY, USA). The cell counting kit-8 (CCK-8) was from Dojindo Laboratories, Kumamoto (Japan). Cell-Light™ Edu Apollo 567 in vitro Kit was purchased from RIBOBIO (Guangzhou, China). The Annexin V-FITC Apoptosis Detection kit was obtained from Keygen Biotech (China). The agarose and CNBr-activated Sepharose™ 4B were from Sigma-Aldrich (Shanghai) Trading Co. Ltd. Transwell Boyden chamber system containing a polycarbonate filter (6.5 mm in diameter, 8 μm pore size) was from Corning Life Sciences. The polyvinylidene fluoride (PVDF) membrane was from Millipore (USA). The antibody was purchased from Cell Signaling Technology (CST).

Cell culture

The majority of methods were described in our previous study [16]. NPC cell lines including CNE2 and HNE1 cells were grown in RPMI-1640 medium containing 10% FBS. Immorta-lized nasopharyngeal epithelial NP69 cells were fostered in Keratinocyte-SFM containing 2% FBS. All cells were cultured at 37°C in an incubator with 5% CO$_2$ and saturated humidity. Cells stayed at the logarithmic phase were used for the following experiments.

Cell counting kit (CCK)-8 assay

Logarithmically growing cells (2 × 10^3) were seeded in 96-well plates. After cells attached to the wall of the plates, they were treated with different concentrations of SAA (0, 10, 20 and 40 μM). After another 24 and 48 h of culturing, CCK-8 was added to wells (at a final concentration of 10%) and incubated for a further 3 h at 37°C. The absorbance was measured by a spectrophotometric microtiter plate reader (Dynatech Laboratories, Alexandria, VA) at 450 nm. The experiment was performed in triplicate.

EdU proliferation assay

CNE2 and HNE1 cells (2 × 10^3) were seeded in 96-well plates and incubated under standard conditions in complete medium for 24 h. The culture medium was then changed to RPMI-1640 containing 2% FBS with SAA in different concentrations (0, 10, 20 and 40 μM). After incubated for 48 h, cells were detected using the incorporation of 5-ethynyl-2'-deoxyuridine (EdU) with the Cell-Light™ Edu Apollo 567 in vitro Kit. Briefly, the cells were incubated with 50 μM EdU for 2 h. Then according to the manufacturer's instructions, Apollo staining was performed to examine the cells. The proportion of cells that incorporated EdU was determined by fluorescence microscopy. The experiment was performed in triplicate.

Foci-formation assay

For foci-formation assay, CNE2 and HNE1 cells (500 cells/well) were seeded in 6-well culture plates overnight. The culture medium was then replaced with complete medium containing different concentrations of SAA (0, 5, 10, 20 and 40 μM) every 3 days. After 2 weeks of incubation, the cells were gently washed twice with PBS, then fixed with methanol for 15 min, and stained with 0.5% of crystal violet. Colonies containing greater or equal to 50 cells were manually counted. The experiment was performed in triplicate.

Soft-agar colony formation assay

The soft-agar colony formation assay was conducted to detect the cellular transformation *in vitro*. The lower gels (with the same volume of 2 × RPMI-1640 medium and 1.2% agarose) were added to the 6-well culture plates, and incubated in room temperature. And it took several minutes to solidify the lower gels in the room temperature. CNE2 cells (1000 cells/well) mixed with 0.4% semi-solid agar (with the same volume of 1 × RPMI-1640 medium, 2 × RPMI-1640 medium and 1.2% agarose) containing different concentrations (0, 5, 10, 20 and 40 µM) of SAA were plated on the lower gels. 100 µl of culture medium containing different concentrations of SAA was added to the gels every 3 days to guarantee the nutrition and pesticide effect. Colonies including more than 100 cells were counted for statistics. The experiment was performed in triplicate.

Wound healing assay

The wound healing assay was for probing collective cell migration. CNE2 and HNE1 cells (5 × 10⁵) were seeded to the 6-well culture plates for 24 h. The wounds were created by 20 µl tips, and washed with PBS twice. Pictures of the wounds were captured under inverted microscope. Serum-free media with different concentrations (0, 10, 20 and 40 µM) of SAA were added to wells respectively. Pictures were captured after incubating for 12 h and 24 h. The experiment was performed in triplicate.

Transwell Boyden chamber assay

Logarithmically growing cells were treated with trypsin without EDTA and resuspended in serum-deprived medium. The cell suspension (1 × 10⁵ cells/well for invasion assay, 3 × 10⁴ cells/well for migration assay) containing different concentrations of SAA (0, 10, 20 and 40 µM) was added to the upper chamber (chambers were pre-coated with matrigel for invasion assay). In the lower chamber, RPMI-1640 medium containing 10% FBS was added as a chemo attractant. The cells were incubated for 48 h at 37°C in an incubator. The system was washed with PBS twice. Cells on the top surface of the chambers were removed with cotton swabs. Membranes were then fixed with methanol for 15 min at room temperature, and stained with 0.5% crystal violet for 2 h. Five visual fields

were randomly selected to be photographed under a light microscope at 200 × magnification. The experiment was performed in triplicate.

Annexin V-FITC/PI double staining

Logarithmically growing cells (1 × 10⁵ cells/well) were seeded in 6-well plates. After cultured for 24 h, the medium was replaced with RPMI-1640 medium containing different concentrations of SAA (0, 10, 20 and 40 µM). After incubated for 48 h, cells were collected and washed with PBS twice. After resuspended in binding buffer, cells were stained with Annexin V-FITC and PI subsequently. Samples were incubated in the dark for at least 15 min prior to analysis using flow cytometry. The experiment was performed in triplicate.

In vitro pull down assay

CNBr-Sepharose 4B beads were suspended with 1 mM HCl medium and centrifuged at a low speed for 3-5 times, and then mixed with the same volume of SAA or ddH₂O (solvent of SAA, act as control group) in coupling buffer (0.1 M NaHCO₃, pH 8.3, 0.5 M NaCl) and rotated slowly at 4°C overnight. The beads were washed to remove excess SAA with coupling buffer at least five times, followed by incubated in 0.1 M Tris-HCl buffer (pH 8.0) to block remaining active groups for 2 h. Then the beads were washed at least three cycles with 0.1 Macetate buffer (pH 4.0) containing 0.5 M NaCl and 0.1 M Tris-HCl containing 0.5 M NaCl (pH 8.0). Both SAA-Sepharose 4B and ddH₂O-Sepharose 4B were combined with the cellular supernatant fraction of CNE2 cells (600 µg) in reaction buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% NP-40, 2 µg/mL of BSA, 0.02 mM PMSF, 1 × protease inhibitor cocktail). The beads were gently rotated at 4°C overnight, and then washed with washing buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% NP-40, and 0.02 mM PMSF) five times. Proteins bound to the beads were eluted with protein buffer and then examined by Western blot.

Western blot assay

Western blot assay was conducted to monitor whether SAA affects the expression level of proteins that were substrates of Pin1. CNE2
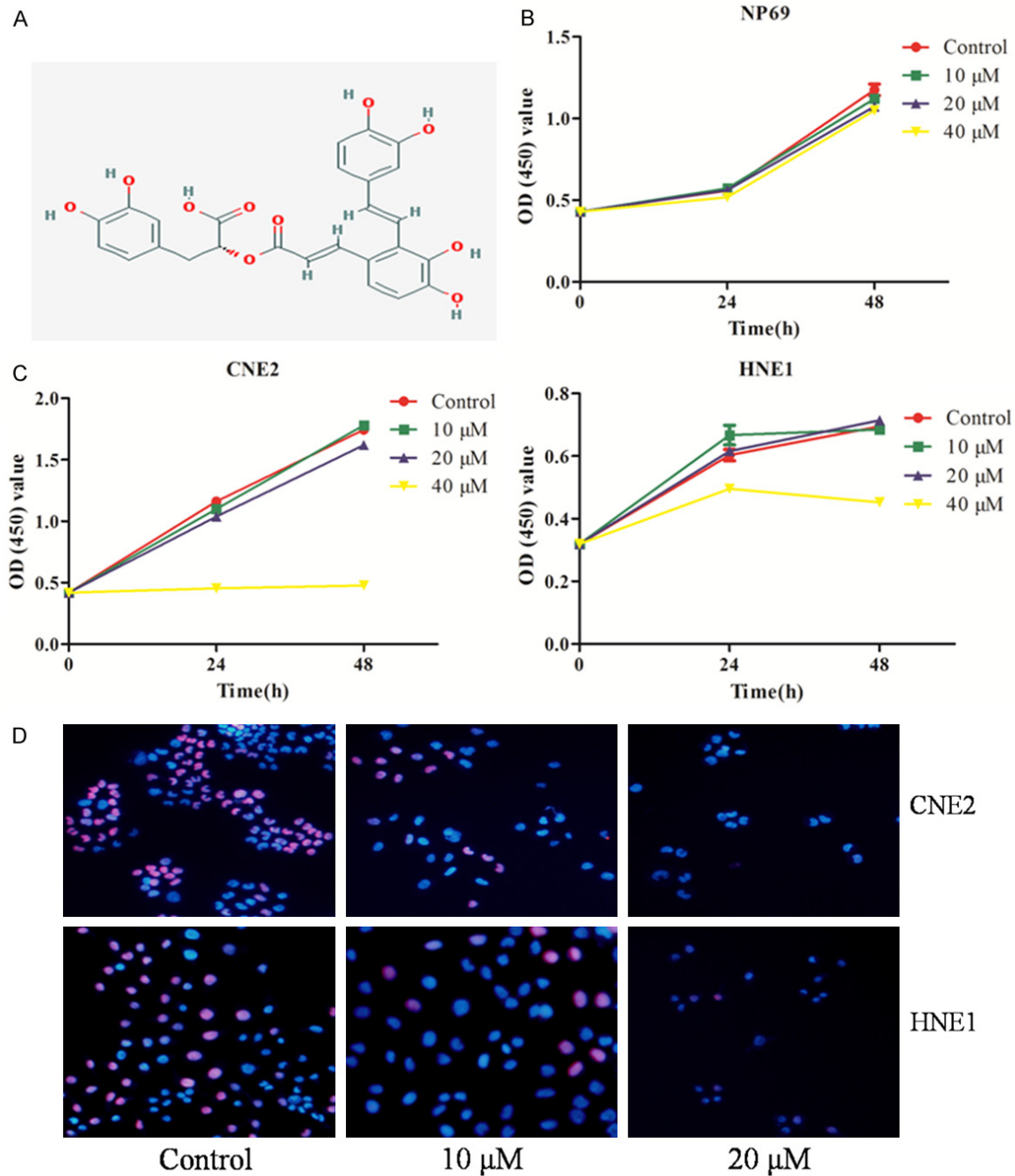
Figure 1. Effects of SAA on cell growth of nasopharyngeal carcinoma (NPC) cells and immortalized nasopharyngeal epithelial cells NP69. A. Structure of SAA is obtained from PubChem Compound database. B. NP69 cells (2×10^3 cells/well) were seeded in 96-well plates and treated with different concentrations of SAA (0, 10, 20 and 40 μM) and absorbance was examined using the CCK-8 assay every 24 h. C. CNE2 and HNE1 cells (2×10^3 cells/well) were seeded in 96-well plates and treated with different concentrations of SAA (0, 10, 20 and 40 μM) and absorbance was examined using the CCK-8 assay every 24 h. D. CNE2 and HNE1 cells were firstly treated with SAA for 48 h. Then cells were stained by Apollo reaction buffer and Hochest reaction buffer, respectively. Finally, cells were observed by fluorescence microscope.

cells (1×10^6 cells/well) were seeded in 6 cm dishes incubated for 24 h and then treated with different concentrations (0, 10, 20 and 40 μM) of SAA for 48 h. The cells were disrupted in 150 μl lyses buffer. Proteins were collected and their concentrations were measured by the
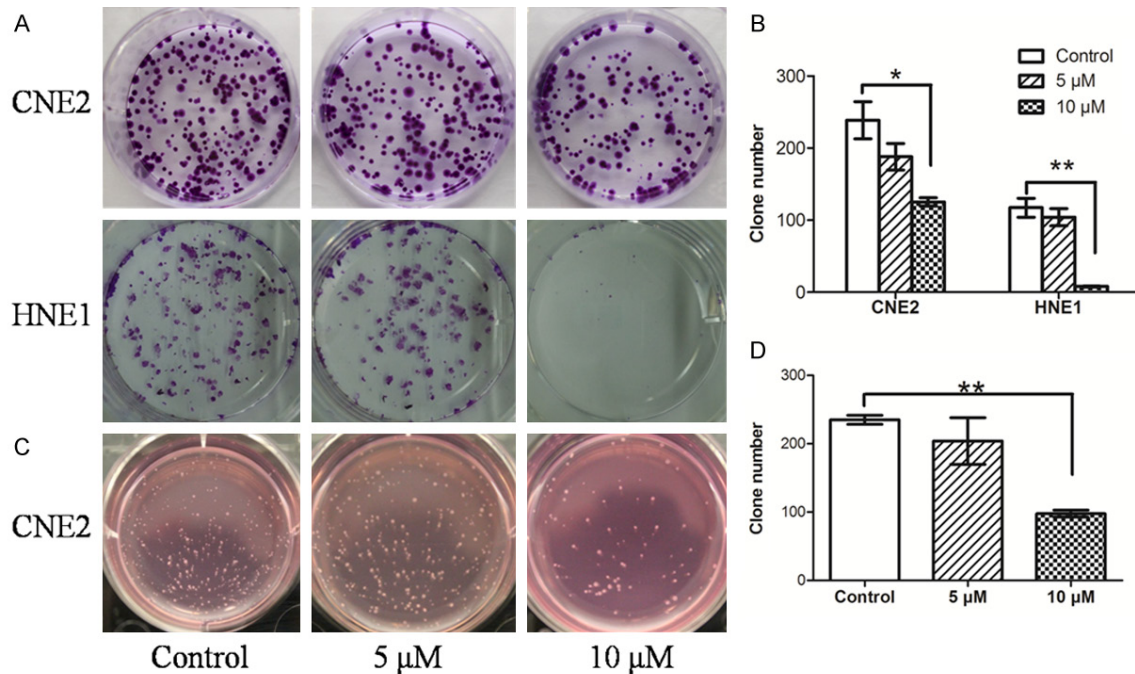
Figure 2. Effects of SAA on colony-forming ability of CNE2 and HNE1 cells. A. Cell colony formation was examined using the colony formation assay. B. Data are presented as the mean ± SD for foci-formation experiments, *P < 0.05; **P < 0.01. C. Colony-forming ability of CNE2 cells in soft agar. D. Data are presented as the means ± SD for colony-forming ability of CNE2 cells in soft agar experiments, **P < 0.01.

pierce™ BCA protein assay kit (Thermo Scientific). Equal amount of the extract was subjected to SDS-PAGE, and then transferred to PVDF membrane, and blocked in 5% non-fat milk for 2 h. The membranes were then incubated with primary antibody at 4°C overnight, prior to incubation with the secondary antibody for 2 h. Protein expression level was visualized by LI-COR Odyssey Infrared Imaging System.

Statistical analysis

All experiments in this study were replicated for at least three times. And the values in this article were exhibited as mean ± standard deviation (SD). All statistical analyses and graph drawing were performed by Graphpad Prism (GraphPad Software, Inc. USA). All the comparisons between treated groups and the control group were performed by using Two-tailed Student's t-test if appropriate. P < 0.05 was considered to be statistically significant.

Results

SAA inhibits the proliferation of NPC cells

CCK-8 assay was used to test the cytotoxicity effect of SAA on NPC cell lines and immortal-

ized nasopharyngeal epithelial NP69 cells. The structure of SAA is obtained from PubChem Compound database (**Figure 1A**). The results of CCK-8 assay showed that different concentrations of SAA (10, 20 and 40 μM) could significantly inhibit the proliferation of CNE2 and HNE1 cell lines in a time and concentration dependent manner (**Figure 1C**). At both 24 h and 48 h, a concentration of 20 μM SAA had slight inhibition (*P < 0.05) on the proliferation of CNE-2 cells. As drug concentration increased up to 40 μM, the proliferation of CNE-2 cells was significantly inhibited (*P < 0.01). Similar results were obtained using HNE1 cells. Nevertheless, SAA had nearly no effect on the proliferation of immortalized nasopharyngeal epithelial NP69 cells at the same treatment condition (**Figure 1B**). Edu assay was applied to further test the effect of SAA on NPC cells proliferation. The results indicated that SAA could inhibit the proliferation of NPC cells in a concentration-dependent manner (**Figure 1D**).

Effects of SAA on the colony formation ability of NPC cells

Both foci-formation assay and soft agar colony formation assay showed that SAA could also inhibit the colony formation ability of NPC cells
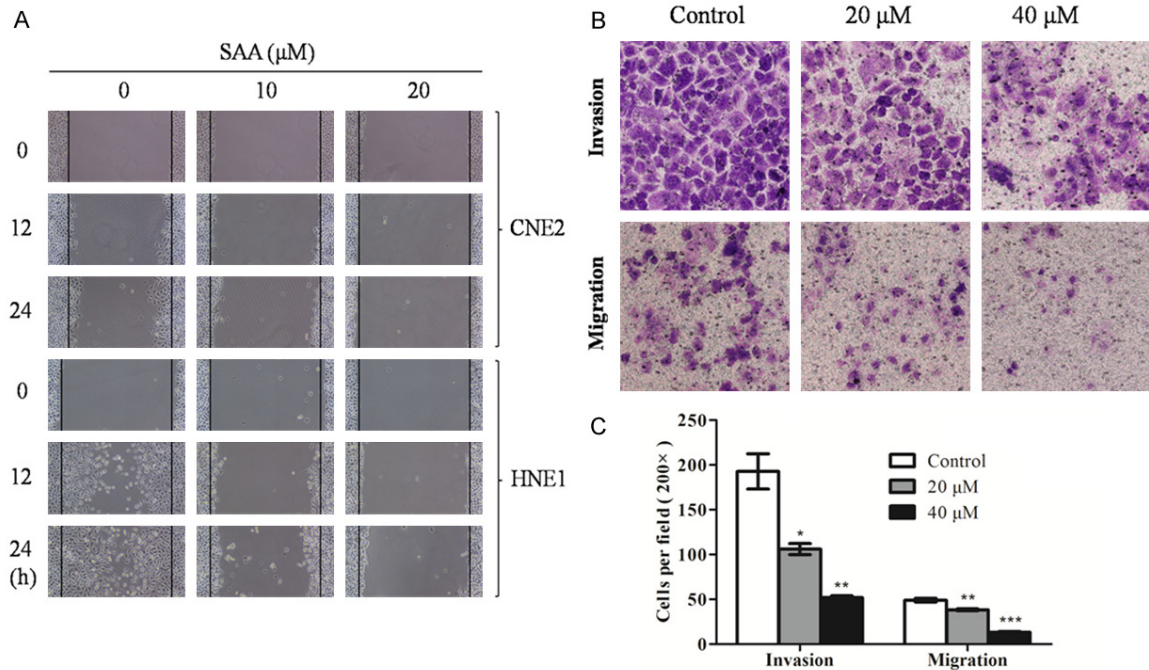
Figure 3. SAA inhibited the invasion and migration ability of CNE2 and HNE1 cells. A. CNE2 and HNE1 cells were treated with different concentrations (0, 10, 20 and 40 µM) of SAA to observe their migration ability. B. Transwell Boyden chamber assays were performed to compare the invasion and migration potential of SAA at different concentrations (at 200 × magnification). C. The total numbers of cells in CNE2 cell line were counted under a microscope in five predetermined fields (mean ± SD, n=3, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

in a concentration dependent manner. For foci-formation assay, the colony number in the group exposed to different concentrations of SAA (0, 5 and 10 µM) in CNE2 cells was 238.7±25.8, 188.0±18.5, and 125.3±5.8, respectively. In HNE1 cells, the colony number was 1173±13.1, 104.0±12.0, and 8.0±0.6, respectively (**Figure 2A** and **2B**). At 10 µM, the colony formation abilities of both CNE2 cells and HNE1 cells were significantly inhibited by SAA (*$P < 0.05$, **$P < 0.01$). For soft agar colony formation assay, the colony number in the group exposed to different concentrations of SAA (0, 5 and 10 µM) in CNE2 cells was 235.0±3.8, 204.0±19.7, and 97.7±3.0, respectively (**Figure 2C** and **2D**). At 10 µM, the colony formation ability of CNE2 cells was significantly inhibited by SAA (**$P < 0.01$).

SAA affects the migration and invasion potential of NPC cells

Cell invasion and migration are key steps for tumor metastasis. We detected the migration and invasion potential of NPC cells by wound healing assay and Transwell Boyden chamber assay. The results of wound healing assay sh-

owed that different concentrations of SAA (20 and 40 µM) could significantly inhibit the migration ability of CNE2 and HNE1 cell lines (**Figure 3A**). The invasion ability of CNE2 cells detected by Transwell Boyden chamber assay showed that the number of CNE2 cells in the group exposed to different concentration of SAA (0, 20 and 40 µM) invading through the matrigel was 192.7±19.7, 106.0±6.1, and 51.7±2.2, respectively (**Figure 3B** and **3C**). At 20 µM, the invasion ability of CNE2 cells was significantly inhibited by SAA (*$P < 0.05$). The migration ability of CNE2 cells detected by Transwell Boyden chamber assay showed that the number of CNE2 cells in the group exposed to different concentration of SAA (0, 20 and 40 µM) migrating through the polycarbonate membrane was 49.0±2.1, 38.3±0.9, and 13.3±0.7, respectively (**Figure 3B** and **3C**). At 20 µM, the migration ability of CNE2 cells was significantly inhibited by SAA (**$P < 0.01$).

Effects of SAA on apoptosis of NPC cells

We tried to investigate whether the proliferation inhibiting effect of SAA on NPC cells was caused by the promotion of apoptosis. The
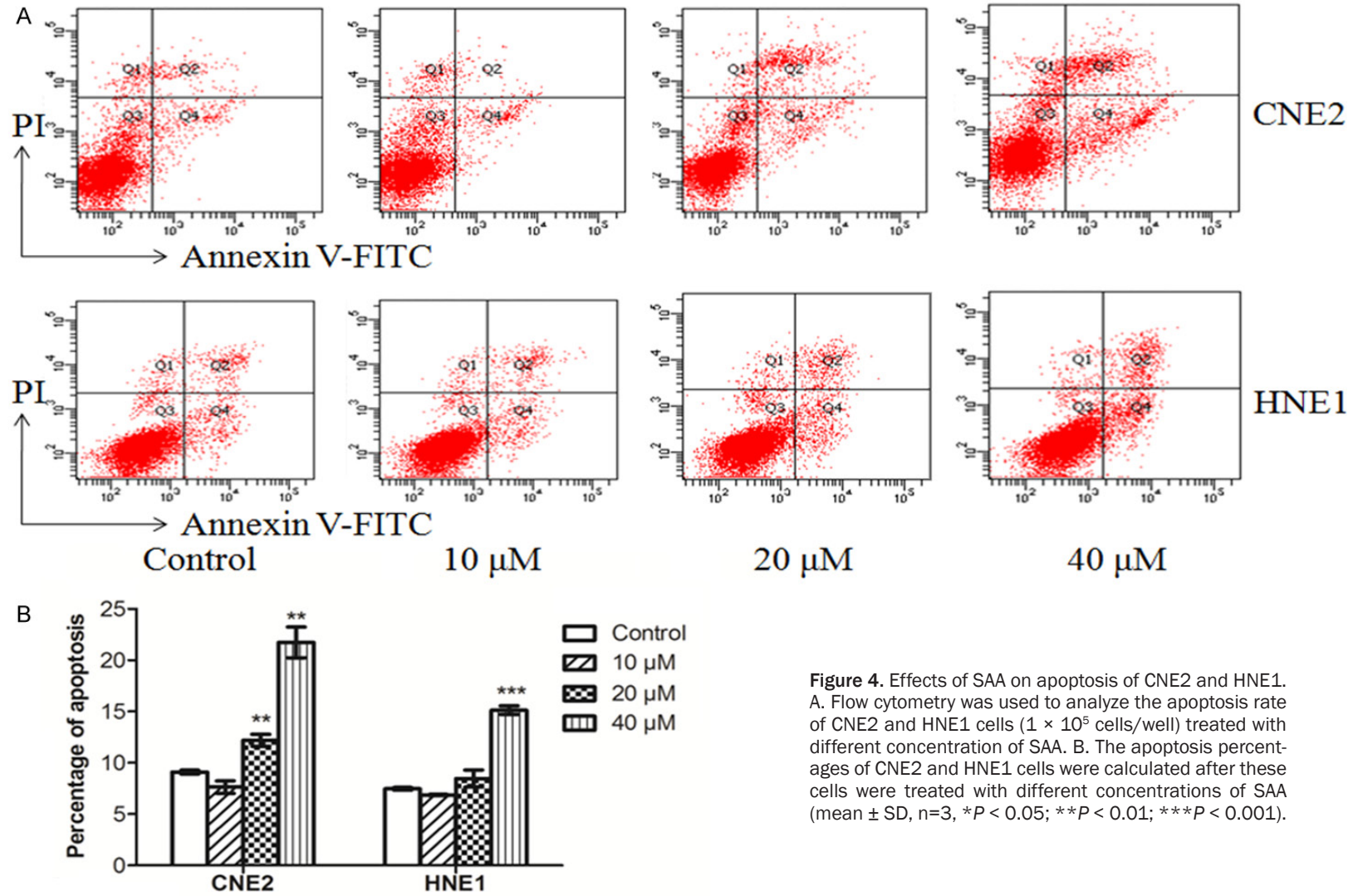
Figure 4. Effects of SAA on apoptosis of CNE2 and HNE1. A. Flow cytometry was used to analyze the apoptosis rate of CNE2 and HNE1 cells (1×10^5 cells/well) treated with different concentration of SAA. B. The apoptosis percentages of CNE2 and HNE1 cells were calculated after these cells were treated with different concentrations of SAA (mean ± SD, n=3, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$).
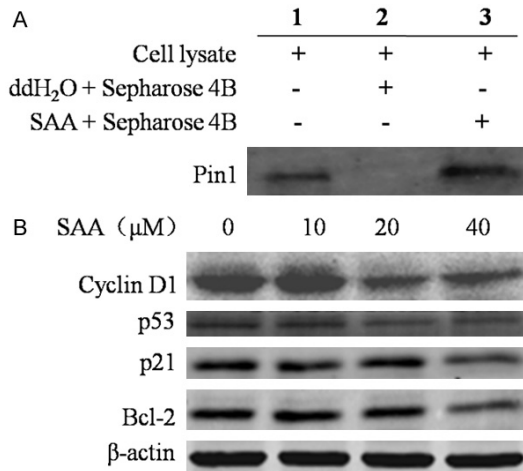
Figure 5. SAA binds with Pin1 and modulates its substrates. A. SAA was bound with Pin1. Lanes 1 showed the total protein of cells. Lanes 2 and lanes 3 showed the solvent group and SAA group respectively. B. SAA down-regulated the protein levels of cyclin D1, p53, p21 and Bcl-2.

results indicated that the apoptosis rate of CNE2 cells exposed in different concentration of SAA (0, 10, 20 and 40 µM) was 9.1%, 7.6%, 12.2% and 21.7%, respectively. In HNE1 cells, the apoptosis rate was 7.5%, 6.9%, 8.5% and 15.1%, respectively (**Figure 4A**). The apoptotic rate of CNE2 cells was significantly higher at 20 µM group than its control group (**Figure 4B**, *$*P < 0.05$). However, statistically insignificant result was found at 20 µM group on HNE1 cells. At the concentration of 40 µM, the apoptosis of CNE2 and HNE1 cells was both dramatically increased compared with the control group, respectively (**Figure 4B**, $**P < 0.01$, $***P < 0.001$).

SAA binds directly with Pin1

Pin1 is a peptidylprolyl isomerase (PPIase) that plays an important role in cancer development. The *in vitro* pull down assay was applied to investigate whether Pin1 could be a direct target of SAA. The result demonstrated that Pin1 was directly bound with SAA (**Figure 5A**, lane 3). The total proteins of CNE2 cells were loaded as input (**Figure 5A**, lane 1) and no Pin1 protein was found in the solvent (**Figure 5A**, lane 2).

SAA reduced the expression of Pin1 substrates

The protein level of several Pin1 substrates, such as cyclin D1, p53, p21 and Bcl-2 could be modulated by Pin1 [17-21]. Western blot was

used to detect whether the protein level of these Pin1 substrates was affected by SAA. The results demonstrated that SAA could down-regulate the proteins level of cyclin D1, p53, p21 and Bcl-2 (**Figure 5B**). The de-regulation of these proteins was obvious when SAA was at high concentration.

Discussion

Approximately 86,700 new cases and 50,800 deaths of NPC occurred in 2012 worldwide [1]. The tolerance of radiation therapy [22] and the multi-drugs resistance [23] are the principal cause of the failure in anti-tumor therapy. Along with the development of medical technology, the survival rate of patients with NPC is increasing. However, NPC remains to be a devastating malignancy because there is no effective therapy for patients with recurrence and metastasis. Therefore, it is a matter of great concern for researchers to find out more target-specific anti-tumor drugs which are of high efficiency, low toxicity and preferable curative effect.

In the past several decades, researchers have demonstrated that natural compounds extracted from traditional Chinese medicine possess strong potential anti-tumor effect. Till now, not a few natural compounds have developed as commonly used anti-cancer drugs such as Paclitaxel and Vinblastine. These Chinese herbal medicines have even taken over a dominant percentage of the clinical therapy market [24, 25]. Salvianolic acid A (SAA) is a compound extracted from *Salvia miltiorrhiza* which possesses a wide range of biological properties [26, 27]. As a matter of fact, SAA performs well in the treatment of cardiovascular diseases. In recent years, many investigators have been interested in studying the antitumor activity and the potential anti-tumor mechanisms of SAA. Finally, SAA has been shown to induce apoptosis [9] and inhibit metastasis [28] in various cancer cell lines. Recently, SAA was found to inhibit the proliferation of several tumor cell types as well [29, 30]. In our study, it was shown that SAA markedly inhibited the proliferation and clone-forming ability of CNE2 and HNE1 cells. SAA had nearly no cytotoxicity on the proliferation of immortalized nasopharyngeal epithelial NP69 cells, which means that SAA was a safe compound. SAA was also reported to inhibit the migration and invasion in breast cancer cells by inactivating transgelin-2

protein [15]. Our results further indicated that SAA significantly decreased migration and invasion potential of CNE2 and HNE1 cells in a dose and time-dependent manner. While Metastasis and invasion were the major causes of NPC mortality [31], SAA was a promising candidate compound for the treatment of NPC.

The most recent studies indicate that Pin1 plays an important role in the transformation and oncogenesis of carcinoma cell [17], and may be a protein target for drug treatment of tumor. It is an approximately 18 kDa protein composed of two functional domains: the N-terminal WW domain (1-39 amino acids) and the C-terminal PPIase domain (45-163 amino acids). Pin1 selectively recognizes phosphorylated Ser/Thr-Pro (pSer/Thr-Pro) peptide sequences and catalyzes the conformation of substrates after their phosphorylation to further control protein function [32-36]. For example, Pin1 regulated Akt stability and phosphorylation through the phosphorylated Thr-Pro motifs of Akt [37]. It was reported that the minor genotype of Pin1 promoter was associated with decreased risk of NPC in a Chinese population [38]. The potential anti-tumor mechanism of SAA was further explored in our study. Our results revealed that SAA might exert its anti-tumor activity through the specific binding to Pin1. Furthermore, our data indicated that SAA down-regulated the protein expression of Cyclin D1, P53, P21 and Bcl2, which were the most important Pin1 substrates [17]. P53 [39], P21 [40] and Bcl2 [41] are well-known apoptosis related proteins. The SAA affecting expression of these proteins could further elucidate the apoptosis found in NPC cells treated with SAA.

It was reported that SAA could not only up-regulate the phosphorylation of PTEN protein level [11], but also markedly down-regulate the protein expression levels of Src phosphorylation (phospho-Src), Akt phosphorylation (phospho-Akt), and NADPH oxidase 4 (Nox4) in a concentration-dependent manner [42]. SAA could reverse the paclitaxel resistance by suppressing transgelin 2 [15] or attenuating PI3K/Akt pathway [13]. Besides, S-3-1, a synthetic intermediate of a SAA derivative, existed strong functional activity to suppress the growth of mouse tumors through the inhibition of the function of Ras oncoprotein, the up-regulation of expression of P53 tumor suppressor gene

and the interruption of P46-associated mitogen-activated pathway [43]. Our data indicated that peptidylprolyl isomerase Pin1 was a potential target of SAA.

In brief, our research indicated that SAA suppressed the proliferation and colony formation ability, migration and invasion potential of CNE2 and HNE1 cells, bound with Pin1 and promoted apoptosis by affecting the Pin1 signaling pathway. Our results implied that SAA may be a potential drug for nasopharyngeal carcinoma.

Disclosure of conflict of interest

None.

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